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TITLE: The Role of Growth Hormone and Insulin-Like Growth Factor-1
in Human Breast Cancer Growth in a Mouse Xenograft Model

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13. ABSTRACT (Maximum 200 words) <p>The purpose of this research is to determine the role of human growth hormone (hGH) and insulin-like growth factor 1 (IGF-1) in the development of an immunodeficient mouse model for human breast cancer. Human GH and IGF1 may be critical to the initiation and progression of tumor growth <i>in vivo</i>.</p> <p>Results suggest that it is questionable whether rhGH alone or in addition to estrogen has a significant role in the development of a primary tumor or the progression of tumor growth in the animal model. In addition, growth hormone may be semi-inhibitory to growth for tumors dependent upon estrogen. Exogenous IGF1 however, enhances the time to development of a palpable primary tumor and likely has a role in sustaining tumor growth and size over and above what has been achievable with estrogen alone. The effect of human rhGH and IGF1 on tumor IGF1, IGF2 and IGFR is currently under evaluation in this laboratory on tumor specimens obtained from the experimental animals. Over the next year in this laboratory, primary tumors from patients under care at Maine Medical Center, will be placed into the <i>scid/scid</i> mouse model and supplemented with IGF1 to establish if our preliminary results can be applied to the development of new xenograft models.</p>				
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Yue F. Wang MD 12/4/95

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Statement of Work

FINAL REPORT for Grant DAMD17-97-7309

1.0 INTRODUCTION:

1.1 Subject:

Specific genes within the immune and endocrine systems are likely to be the major controlling elements in the successful development of mouse models for mammary tumor xenografts. We believe that growth factors, specifically human growth hormone (hGH) and Insulin Like Growth Factor (IGF1) may be critically important in the successful establishment of such xenografts in an animal model.

1.2 Purpose:

The purpose of this research is to determine the role of hGH and IGF-1 in the development and maintenance of an immunodeficient mouse model for human breast cancer.

1.3 Scope

Human breast cancer growth in animal models is dependent upon an intact GH/IGF-1 axis. Based upon our preliminary data, we believe that hGH may be critical to the initiation of a primary breast neoplasm *in vivo*. IGF-1 may be critical to maintaining tumor growth *in vivo*. When the GH/IGF-1 axis is interrupted or impaired, tumor growth may become more directly influenced by 17- β estradiol.

To test the hypothesis, the following sets of experiments have been executed:

Experiment 1/Specific Aim 1: Determine the amount of recombinant human growth hormone (rhGH) that needs to be administered to the experimental animal to result in (i) early engraftment of palpable tumors and (ii) accelerated growth of the tumor in the *scid/scid* mouse model, and to correlate serum GH and IGF1 levels with tumor IGF1 and IGFR levels by northern and western analyses. Experiments include administration of rhGH both by continuous infusion and by daily administration to mimic the normal circadian rhythm of human growth hormone.

Experiment 2/Specific Aim 2: To determine the role of IGF1 in the initiation and/or the progression of primary breast cancer growth in a *scid/scid* mouse model and to correlate serum IGF1 with tumor IGF1 and insulin growth factor receptor (IGFR) levels by northern and western analyses.

Experiment 3/ Specific Aim 3: To determine the dose of 17- β estradiol administration critical to tumor engraftment and progression of growth in *scid/scid* mice that have an impaired GH/IGF1 axis and if exogenous 17- β estradiol can further enhance tumor growth in animals administered optimal concentrations IGF1 and/or rhGH.

Experiment 4/Specific Aim 4: To grow primary breast cancer explants in the optimized animal model.

1.4 Background

Development of Animal Models For The Study of Human Breast Cancer:

Since the original report by Rygaard and Povlsen (1) that congenitally athymic nude (*nu/nu*) mice supported the growth of a human colon adenocarcinoma following subcutaneous injection, these T cell-deficient animals have been utilized as experimental hosts for a great variety of human neoplasms. However, there has been only limited success in utilizing *nu/nu* mice as hosts for primary human breast carcinomas. In an extensive study of 262 infiltrating ductal carcinomas, Giovinella et al (2) found that only 6.1% of such primary carcinomas could be grown in *nu/nu* mice following subcutaneous injection. Moreover, the human breast carcinomas that did grow successfully in *nu/nu* mice commonly failed to display metastatic properties (3). It has been suggested that the variability in success of metastatic human tumor growth in *nu/nu* mice may be due to background modifying genes (4) that may influence the growth of the human tumors or the metastasis of such tumors. Since there has been only limited success in growing human breast tumors in *nu/nu* mice, preliminary experiments have examined the growth of such tumors in C.B.17 mice homozygous for the severe combined immunodeficiency (*scid*) mutation. C.B17-*scid/scid* mice lack T as well as B cells. Initial data are promising since cell line derived human breast carcinomas show increased take rates and grow faster in *scid/scid* mice than in *nu/nu* mice (5). However, such studies have been limited to the C.B17-*scid/scid* mouse. An added benefit to establishing a breast cancer model in this animal is that the *scid/scid* mouse can have its bone marrow reconstituted with human hematopoietic cells. This feature of the *scid/scid* mouse would allow this animal model to be used in experiments studying the role of human growth factors and cytokines in supporting or impairing human primary tumor growth and the process of metastasis. Use of non-obese diabetic (NOD) *scid/scid* mice may prove to be a superior animal for such experimentation due to impaired natural killer (NK) cell activity in addition to impaired B and T cell function.

The Role of Human Growth Hormone In Human Breast Cancer: A variety of growth factors have been identified that are mitogenic for breast cancer cell lines *in vitro*. The focus of this experimental work is to establish if alterations in the hGH/IGF-1 axis can be made that facilitate the engraftment and subsequent growth of a primary human breast cancer explant in an immunodeficient mouse model. Focus on the hGH/IGF-1 axis in the experimental animals is selected as an area of importance based upon the results of recent experimental results reviewed below. Endocrine glands providing estrogen, progesterone, glucocorticoid, and insulin are prominent regulators of mammary tissue growth. Moreover the protein hormones of the human lactogenic series - pituitary prolactin (PRL) and growth hormone (GH) plus placental lactogen (PL) are of

unique importance because of their species specific biological properties (6). GH has been implicated as a growth factor for human breast cancer (7) and it has been shown that rhGH stimulates breast cancer growth through IGF-1 and possibly other growth factors (8). *In vitro*, insulin growth factor receptor (IGF-R), IGF-1, IGF-2 and insulin have all been shown to be mitogens of MCF-7 breast cancer cells (9). The mechanism of this perturbation is unknown, however, it is known that insulin is capable of altering the cell cycle kinetics of MCF-7 human breast cancer cells by facilitating their transit through the G1 phase of the cell cycle (10). *In vitro* it has been shown that estrogen and progesterone may alter the growth of breast cancers by regulating the insulin growth factor binding proteins (IGFBP) and thereby change the carcinoma's responsiveness to IGF-1 (11). In human studies, hGH (7) and IGF-I has been shown to be elevated (12) in operable patients with breast cancer in comparison to uneffected control patients and hGH, IGF-1, IGF-2, and IGF-R levels may be indicators of prognosis or response to treatment (13,14). In another study however, experimental results suggested that in postmenopausal women with breast cancer, the plasma sex steroids fail to influence the concentrations of IGF-1 or IGFBP-1 when present in physiologic concentrations (15). Tamoxifen, an estrogen receptor blocking drug widely used in the adjuvant, metastatic and preventive management of breast cancer has been shown to have a role in the regulation of the GH axis (16). Tamoxifen decreases serum hGH and IGF-I serum levels in treated patients as well as reduced IGF-I in target organs by a mechanism that is pituitary independent (16). These studies all seem to suggest that GH and insulin growth factor(s) may be critical to the establishment of an optimal milieu for the initiation and promotion of breast neoplasia in a patient.

In *in vitro* studies, IGF1 has been demonstrated to have a preferential proliferative effect on estrogen receptor (ER) positive cells in contrast to ER negative cells. Positive correlations have been made between IGFR and ER expression in breast cancer specimens and this presumably reflects the regulation of the IGFR by estrogen in breast tissue (17). IGF receptor activity in human breast cancer is also relevant to breast cancer development. There are two receptors important in IGF1 and IGF2 signaling. The IGF1 receptor is a tyrosine kinase receptor that signals through the ras/raf/MEK/MAPK and accepts IGF1 and IGF2 as ligands that result in cell proliferation. The IGF2 receptor is identical to the mannose-6-phosphate receptor. This receptor translocates proteins containing mannose-6-phosphate moieties and IGF2 to lysosomes for degradation. Mutations in this receptor have been correlated with enhanced proliferation thus implicating this receptor as a tumor suppressor gene in cancers (18,19). Recently it has been established that interruption of the ability of the IGFR to signal using dominant/negative receptors to IGFR is correlated with inhibition of adhesion, invasion and metastasis of breast cancer (20-22). These studies all seem to suggest that IGFs may be critical to the establishment of an optimal milieu for the initiation and promotion of breast neoplasia.

Animal models available for the study of IGFs and their receptors: The *lit/lit scid/scid* mouse has been developed that is simultaneously immunodeficient and

isolated murine growth hormone mGH/IGF1 deficient (23). The little (*lit*) mutation renders the pituitary GH releasing hormone receptor non-functional, and the homozygous mouse is therefore severely deficient in both mGH and mIGF1 (24,25). This immunodeficient, murine GH deficient, murine IGF1 deficient mouse provides an excellent animal model in which human breast xenografts can readily grow and evaluation of the importance of human IGFs can be assessed in xenograft maturation. In this model, Pollak et al have demonstrated that MCF7 tumor cells implanted into *scid/scid lit/lit* mice supplemented with 17- β estradiol develop statistically significant smaller tumors than MCF7 tumors grown in IGF1 replete animals (26). This finding is significant to this proposed work further demonstrating the importance of the presence of IGFs in robust and healthy xenograft growth in this experimental model.

2.0 BODY OF WORK

2.1 METHODS:

Specific Aim 1: Determine the amount of rhGH that needs to be administered to the experimental animal to result in (1) early engraftment of palpable tumors and (2) accelerated growth of the tumor in the *scid/scid* mouse model, and to correlate serum GH and IGF-1 levels with tumor IGF-1 and IGF-R levels by northern and western analyses. Experiments include administration of rhGH both by continuous infusion and by daily administration to mimic the normal circadian rhythm of human growth hormone.

Establishment of MCF7R mouse models

The MCF7R human breast cancer cell line was used in these experiments. MCF7R cells are derived from the parental cell line MCF7. MCF7R cells are rendered resistant to chemotherapeutic drugs due to up-regulation of the multiple drug resistant gene 1 (*mdr-1* gene) and p-glycoprotein. This cell line was established by gradually forcing MCF7 cells resistant to vincristine. It was a gracious gift from Dr. William Hait, Yale University. The animals models were established by injection of 1×10^6 MCF7R cells suspended in Matrigel (Becton Dickinson) into the mammary fat pad of experimental animals 2 days after the initiation of rhGH administration. Animals were assessed weekly for development of tumor growth. Tumors were measured using Vernier caliper. Tumor volumes at each measurement were calculated using the equation

$$v = \pi r^2 l$$

where v is volume, r is the radius of the tumor and l is the length of the tumor.

When tumor growth in experimental and control animals reached 1 X 1 X 1 cm, the animals were euthanized by CO₂ anesthesia, the tumors harvested from the animals, and total RNA extracted.

Selection of experimental animals

Scid/scid mice, *scid lit*^{+/-} mice, *scid/scid lit/lit* and TghGH *scid/scid* mice were used in this experimental aim. NOD *scid/scid* mice served as true experimental control animals. *Scid/scid lit/lit* animals are animals that have inability to produce gonadatropin hormone releasing hormone and also have ineffective production of growth hormone. TghGH *scid/scid* mice are transgenic mice for human growth hormone. *Scid lit*^{+/-} mice are heterozygotes for the *lit/lit* mutation. All animals were obtained from The Jackson Laboratory, Bar Harbor Maine. Dr. Wesley Beamer has developed colonies of TghGH *scid/scid* mice and *scid/scid lit/lit* mice in his laboratories. Funding from this research effort has made it possible to obtain animals from Dr. Beamer.

Administration of recombinant human growth hormone

Mice were divided into two experimental treatment groups. In the first group, recombinant human growth hormone (rhGH) was administered at the onset of the dark cycle of the room in an attempt to approximate the circadian release of growth hormone in the experimental animals. A second set of animals was treated with continuous infusion of rhGH through Alzet miniosmotic pumps. A dose finding study of rhGH administered to *scid/scid lit/lit* mice established that a 10ug rhGH injection into the peritoneal cavity of *scid/scid lit/lit* mice for three consecutive days resulted in serum human growth hormone levels between 1-2.5ng/ml as measured by the Kallestad Quantitope HGH kit (Sanofi Diagnostics, MN). Due to budgetary restraints in this project, the target dose of rhGH of 5ng/ml was financially impossible to achieve.

Group I animals were injected with a daily dose of rhGH of 1.5ug for two weeks and then every other day for the duration of the experiment (12 weeks). Serum IGF-1 levels were determined with IGF1 By Extraction (Nichols Institute, CA) twice during the 12 week experimental period. Group II animals had Alza pumps (model 1002, 0.25ul/hr, 14days) surgically implanted into the subcutaneous tissue on the posterior thorax of the experimental animals and changed every two weeks throughout the duration of the experimental period. The pumps were loaded with 100ul of rhGH, 0.25ug/ml. Serum IGF-1 levels were determined with *IGF1 By Extraction* (Nichols Institute, CA) twice during the 10 week experimental period.

Northern Analysis for IFG1R

Total RNA as well as mRNA was probed with P³² labelled DNA probes specific for IGF1 and IGFR. Probes for IGF1 and IGFR were made from plasmids containing the sequences of interest obtained from ATCC (ATCC, Maryland). Unfortunately, these studies were unsuccessful due to presumably the very low copy number of IGF1 and IGFR. Alternative strategies were developed for their measurement. See next section please.

RT-PCR Assay for IGF1, IGFR and IGF2

For this assay, tumor RNA was extracted using the Tri Reagent (Sigma, St. Louis). Primer sequences for IGF1, IGFR and IGF2 were constructed as per previously published sequences (27). RT-PCR reactions were optimized to produce optimal amplification of the desired targets.

RNA Protection Assay

From our experience with northern analysis, we hypothesized that the sequences that we wished to detect were present in experimental samples in very low copy number. In order to quantify IGF1, IGFR and IGF2 under these conditions, and to also quantitate changes in their copy number under our experimental conditions, an RNA protection assay is in the process of being developed. Probes for the protection assay are the nested PCR products obtained from above for IGF1, IGFR, and IGF2. The PCR products are cut from the gel and gel purified. Using the sense primer only, the PCR product is reamplified, this time with incorporation of P³².

The RNA protection assay is currently being optimized for all three probes. The specific procedures are well detailed (28).

2.1 METHODS: Specific Aim II

Specific Aim II: To determine the role of IGF-1 in the initiation and/or the progression of primary breast cancer growth in a *scid/scid* mouse model and to correlate serum IGF1 with tumor IGF2 and IGFR levels by northern and western analyses.

Establishment of MCF7R mouse models

The MCF7R human breast cancer cell line was used in these experiments. MCF7R cells are derived from the parental cell line MCF7. MCF7R cells are rendered resistant to chemotherapeutic drugs due to upregulation of the multiple drug resistant gene 1 (mdr-1 gene) and p-glycoprotein. This cell line was established by gradually forcing MCF7 cells resistant to vincristine. It was a gracious gift from Dr. William Hait, Yale University. The animal models were established by injection of 1×10^6 MCF7R cells suspended in Matrigel (Becton Dickinson) into the mammary fat pad of experimental animals 2 days after the initiation of rhGH administration. Animals were assessed weekly for development of tumor growth. Tumors were measured using Vernier caliper. Tumor volumes at each measurement were calculated using the equation

$$v = \pi r^2 l$$

where v is volume, r is the radius of the tumor and l is the length of the tumor.

When tumor growth in experimental and control animals reached 1 X 1 X 1 cm, the animals were euthanized by CO₂ anesthesia, the tumors harvested from the animals, and total RNA extracted.

Selection of experimental animals

Scid/scid mice, *scid lit*+/- mice, and *scid/scid lit//lit* mice were used in this experimental aim. NOD *scid/scid* mice served as true experimental control animals. *Scid/scid lit/lit* animals are animals that have inability to produce gonadatropin hormone releasing hormone and also have ineffective production of growth hormone. Because they have decreased production of murine growth hormone, they have ineffective production of murine IGF1 (and likely IGF2). *Scid lit*+/- mice are heterozygotes for the *lit/lit* mutation.

Administration of human IGF-1 to experimental animals

Mice were treated with human IGF-1 (Bachem, CA) by continuous infusion via Alza miniosmotic pumps (Alza pump model number 1002). Prior to beginning the experimentation, a dose finding study of IGF-1 in the *scid/scid lit/lit* was performed. In this experiment it was determined that approximately 2000ng IGF-1 administered daily for three days resulted in a serum level of IGF1 of 128 ng/ml as measured by *IGF-1 By Extraction Kit* (Nichols Institute Diagnostics, CA). The anticipated target dose initially planned upon was 200ng/ml. Because of financial restraints a daily delivered dose approximating a serum value of 65 ng/ml was delivered.

Alza pumps (model 1002, 0.25ul/hr, 14days) were surgically implanted into the subcutaneous tissue on the posterior thorax of the experimental animals and changed every two weeks throughout the duration of the experimental period. The pumps were loaded with 100ul of human IGF1, 50ng/ul. Serum IGF-1 levels were determined with *IGF1 By Extraction* (Nichols Institute, CA) twice during the 10 week experimental period.

Northern Analysis for IGFR

Please see specific details in Specific Aim I.

RT-PCR Assay for IGF1, IGFR and IGF2

See specific details in Specific Aim I.

RNA Protection Assay

See specific details in Specific Aim I.

2.1 METHODS Specific Aim III

Specific Aim III: To determine the dose of 17- β estradiol administration critical to tumor engraftment and progression of growth in *scid/scid* mice that have an impaired GH/IGF-1 axis and if exogenous 17- β estradiol can further enhance tumor growth in animals administered optimal concentrations IGF-1 and/or rhGH.

In order to achieve this goal *scid/scid lit/lit* mice treated with rhGH or IGF-1 were further subgrouped to receive 17- β estradiol or a placebo pellet. Estradiol pellets (Innovative Research of America) were implanted into the subcutaneous tissue of the posterior neck with a trochar. Time to the development of a palpable tumor mass and tumor volume was measured with Vernier calipers and measured as described above. IGF-I and IGF-R levels in experimental tumors is determined by northern and western analyses and compared to levels obtained from *scid/scid lit/lit* mice +/- 17- β estradiol not receiving rhGH or IGF-1 supplementation.

Northern Analysis for IGF1R

Please see detailed procedures in Specific Aim I.

RT-PCR Assay for IGF1, IGFR and IGF2

See detailed procedures in Specific Aim I.

RNA Protection Assay

See detailed procedures in Specific Aim I.

2.1 METHODS: Specific Aim IV

Specific Aim 4: To grow primary breast cancer explants in the optimized animal model.

The goal of this experimental aim is to demonstrate that primary human breast cancer explants can be grown and sustained in the optimized animal model developed in Aims 1-3. Human breast carcinomas are obtained from patients undergoing surgery in the operating suites at the Maine Medical Center and are immediately transferred to the laboratory in Earle's minimal essential medium (MEM) for processing. Samples from each tumor are retained for routine pathologic analysis at Maine Medical Center. In addition, specific notation is made of primary tumor size, nuclear grade, axillary lymph node status, the presence or absence of estrogen and progesterone receptors, ploidy and S-phase analysis (this information is readily available after routine pathologic analysis of the tumor at Maine Medical Center). The tumor is dissected free of necrotic tissue and 2 X 2 mm tumor chunks are cut with a clean scalpel. Experimental animals are anesthetized with 600 μ l intraperitoneal injection of Avertin (1.6 gm

tribromoethanol/ml tertiary amyl alcohol in 80 ml sterile saline). Under sterile conditions, an incision is made in the skin of the chest wall. A tumor chunk is carefully placed in the region of the mammary fat pad. The incision is closed with Clay Adams staples. One week after surgery staples are removed. Animals are checked twice weekly for any evidence of primary tumor engraftment and growth. Tumor measurements and tumor volumes will be scored as described in Specific Aim I.

2.2 RESULTS

Specific Aims IA and III: *In vivo* growth of MCF7R cells in *scid/scid* mice with or without bolus rhGH and 17 β estradiol. : In evaluation of the tumor growth curves displayed in Figures I-III, 17- β estradiol alone is most efficient in stimulating *in vivo* tumor cell engraftment and growth. When rhGH is given in bolus fashion, it appears to inhibit some of the growth stimulatory effects of 17- β estradiol. This is evident most significantly at 5-9 weeks into this study. These observations suggests that rhGH may be stimulating not only the release of growth stimulatory proteins such as IGF1, but a substance(s) that is growth inhibitory. When rhGH is given alone to animals, there is some growth advantage over control animals. This could be due to IGF1 induction or induction of another growth stimulatory protein. It however, can not stimulate MCF7R growth as efficiently as 17- β estradiol alone.

Figure I: The effect of bolus rhGH and 17- β estradiol on MCF7R tumor cell engraftment and growth in NOD *scid/scid* mice

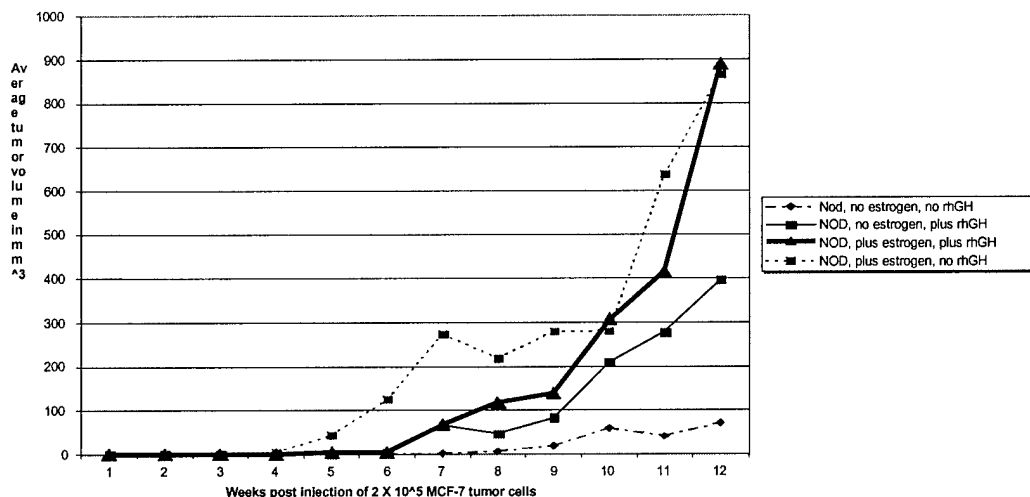


Figure II: The effect of bolus rhGH and 17- β estradiol on MCF7R tumor cell engraftment and growth in *scid/scid lit/lit* mice

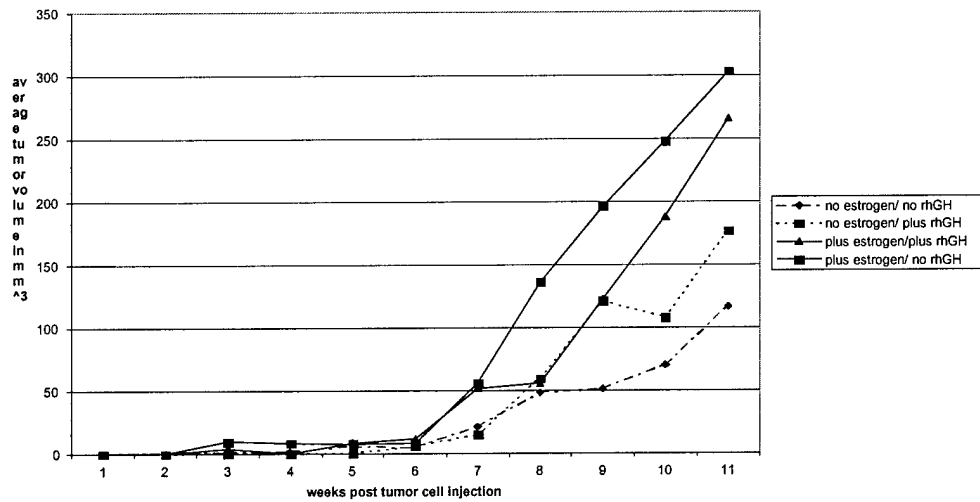
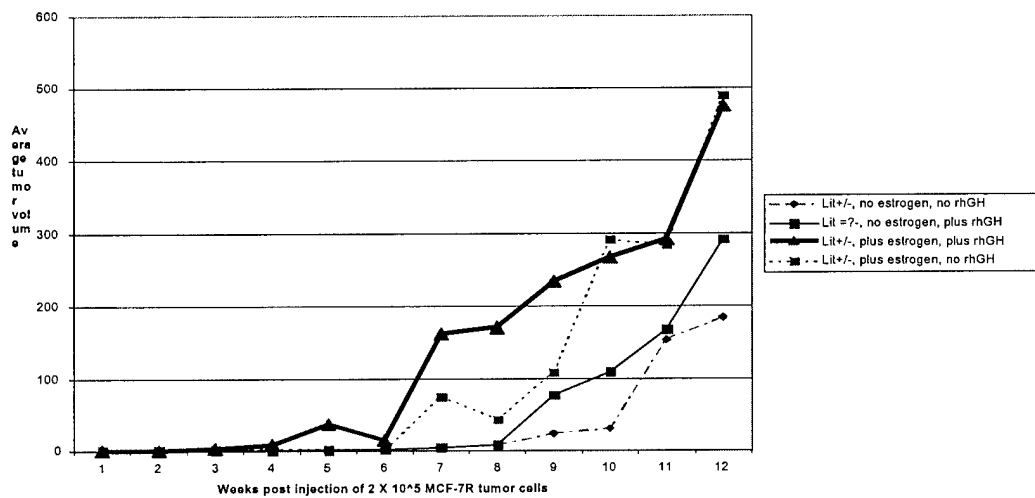


Figure III: The effect of bolus rhGH and 17- β estradiol on MCF7R tumor cell engraftment and growth in *scid/scid lit+/-* mice



Specific Aims IB and III: Continuous infusion rhGH administration and MCF7R tumor cell growth *in vivo*: Data displayed in Figures IV-VI documents the growth of MCF7R breast cancers in immunodeficient *scid/scid* mice exposed to rhGH administered by continuous infusion. The growth hormone was administered through an alza miniosmotic pump placed in the subcutaneous tissue of the mouse. It appears that continuous infusion of rhGH results in no significant alteration of tumor growth in these animal models in comparison to animals treated in the bolus fashion

Figure IV: The effect of continuous infusion rhGH and 17 beta estradiol on MCF-7R growth in NOD *scid/scid* mice

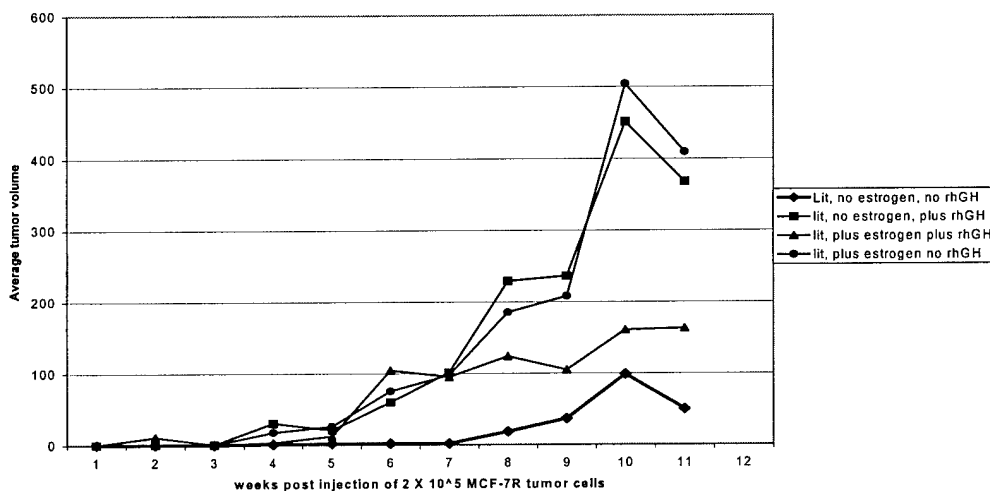


Figure V: The effect of continuous infusion rhGH and 17 beta estradiol on MCF-7R growth in *scid/scid* lit/lit mice

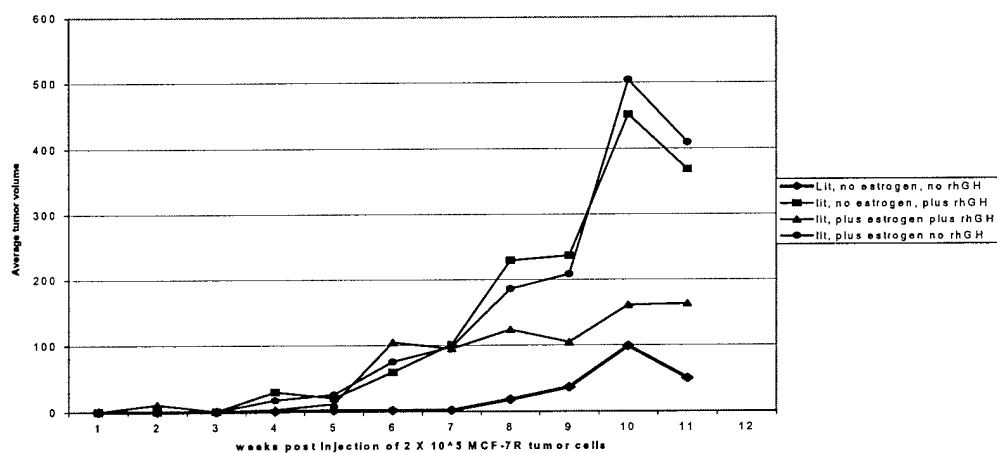
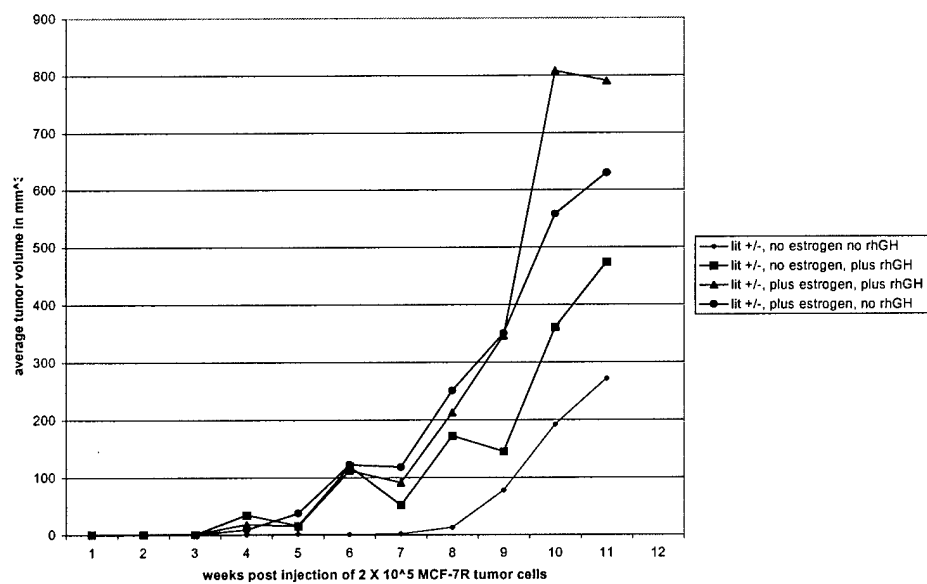
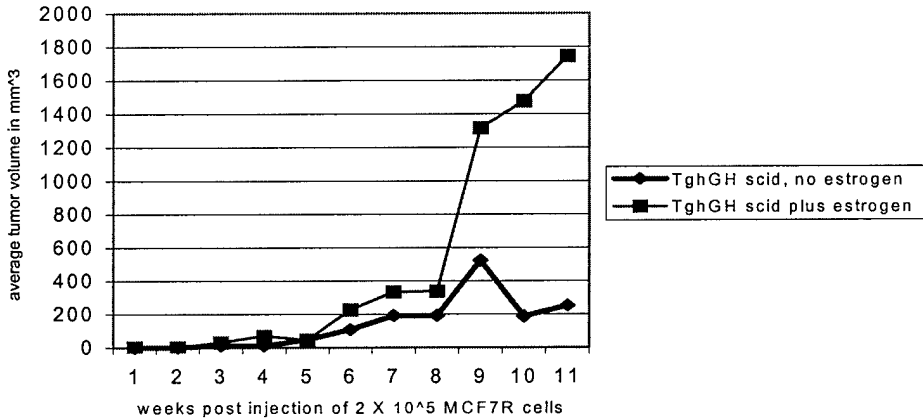


Figure VI: The effect of continuous infusion rhGH and 17 betas estradiol on MCF-7R growth in *scid* lit +/- mice



Specific Aims IB and III : The NOD *scid/scid* mouse transgenic for human growth hormone was also used to establish if human growth hormone alone or in combination with 17- β estradiol could provide a growth advantage for MCF7R breast cancer xenografts. This data is displayed in Figure VII. Transgenic mice bearing these xenografts developed tumors with tumor volumes greater than 1000 mm³ when the TghGH *scid/scid* mice were supplemented with 17- β estradiol. Clearly in this animal model, the presence of human growth hormone is advantageous for enhanced tumor growth and progression.

FIGURE VII: MCF7R tumor growth in TghGH *scid/scid* mice



Specific Aims II and III: To determine the role of IGF-1 in the initiation and/or the progression of primary breast cancer growth in a *scid/scid* mouse model and to correlate serum IGF1 with tumor IGF2 and IGFR levels by northern and western analyses: When *lit/lit scid/scid* animals are supplemented with continuous infusion human IGF1, MCF7R tumors grown in these animals begin to achieve average tumor volumes comparable to xenografts implanted in IGF1 replete animals (29) (Figure VIII) and the time to development of a palpable tumor nodule is significantly decreased. Human IGF1 is therefore a critical endocrine or paracrine growth factor for MCF7R xenografts (as we know that little if any IGF1 is made from the tumor cells themselves) and may have a role in matrix optimization for primary tumor engraftment as well as a role in sustaining tumor growth and size over and above what has been achievable with 17- β estradiol alone. *In vivo*, the addition of 17- β estradiol to IGF1 failed to result in any growth advantage over IGF1 alone. This suggests that in this model system, IGF1 is the more potent and critical growth factor, even in an ER positive cell line. In the absence of hIGF1 however, 17- β estradiol remains the most potent growth factor.

To ascertain if human IGF1 is similarly critical in ER negative xenograft growth, similar experiments were carried out in *scid/scid lit/lit* mice with MDA-MB-231 ER negative cells (Figure IX). These experiments were not included in the original grant proposal, however, we felt that it would be important to establish if the effect of IGF1 on xenograft maturation was unique to only estrogen receptor positive tumors such as MCF7. Although the MDA-MB-231 cells do possess the IGF1R, administration of rhIGF1 to animals bearing these xenografts fails to impart any specific growth advantage over and above animals supplemented with 17- β estradiol or untreated control animals. Although MDA-MB-231 cells possess IGFR, exogenous supplementation of IGF1 failed to result in any up-regulation of the IGFR in comparison to 17- β estradiol treated animals or untreated control animals. This *in vivo* finding continues to support the observations made by others *in vitro* that IGF1 is an important growth factor in tumors that have a responsiveness to 17- β estradiol and that IGF1 likely works in concert with estrogen and the estrogen receptor to control growth of ER positive breast cancers.

Figure VIII: Effect of rhIFG1 administration to *scid/scid/lit/lit* mice (A) and *scid/scid lit/+/-* mice (B) bearing MCF7R xenografts

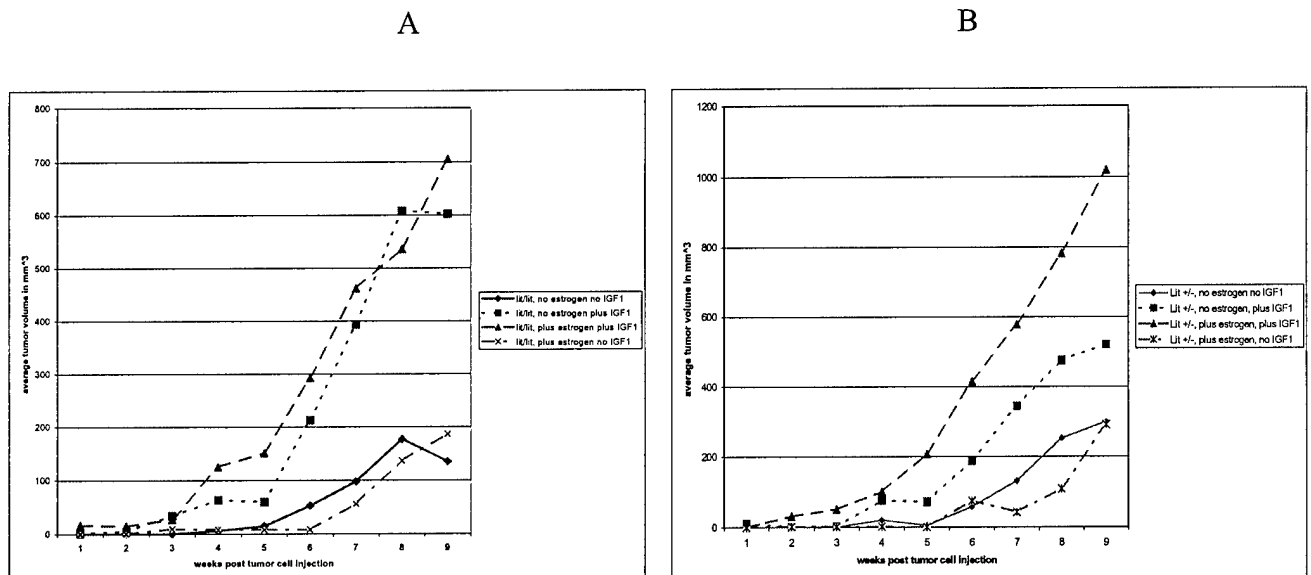


Figure IX: Effect of rhIFG1 administration to *scid/scid/lit/lit* mice (A) and *scid/scid lit/+/-* mice (B) bearing MDA-MB-231 xenografts

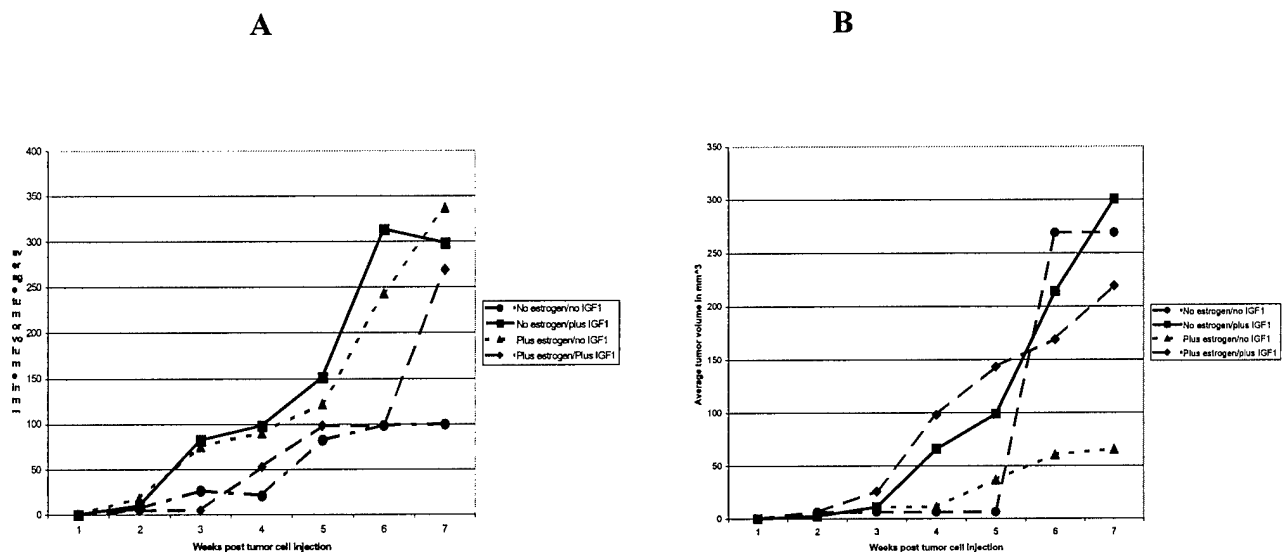
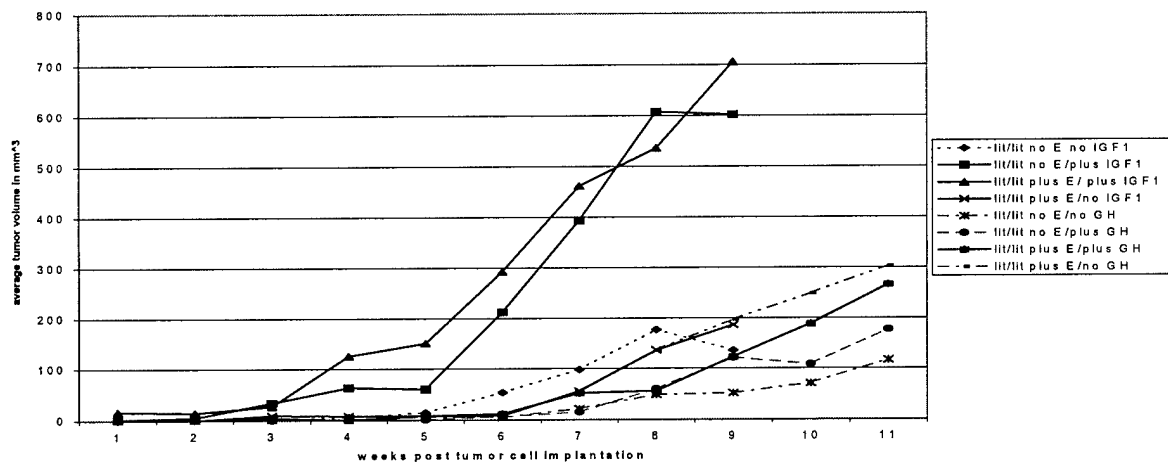


Figure X summarizes the outcome of all experimental conditions evaluated for rhIGF1 and rhGH in *lit/lit scid/scid* mice alone or in combination with 17- β estradiol. RhIGF1 alone is the most important growth factor in this animal model. Supplementation of *lit/lit* mice bearing MCF7R xenografts with IGF1 alone results in tumors that approach the size of tumors grown in *lit +/-* animals or NOD *scid/scid* mice. Also, the time to engraftment of the tumors is 3-4 weeks in comparison to the 6-7 weeks required for tumor engraftment in non IGF1 supplemented animals. When rhIGF1 is present, the effect of 17- β estradiol supplementation is negligible. In the absence of rhIGF1 supplementation, 17- β estradiol is the most critical growth factor. RhGH may play a role in xenograft maturation, however its effect is eclipsed by 17- β estradiol and rhIGF1.

Figure X: MCF7R Tumor growth in *scid/scid lit/lit* mice treated with estrogen, rhGH or IGF1



Specific Aim II: To determine the role of IGF-1 in the initiation and/or the progression of primary breast cancer growth in a *scid/scid* mouse model and to correlate serum IGF1 with tumor IGF2 and IGFR levels by northern and western analyses.

RT-PCR assays for IGF1, IGF2 and IGFR: When cell lines of experimental interest have been assayed in this laboratory by the reverse transcriptase polymerase chain reaction (RT-PCR) for the presence or absence of IGFs and the IGF1R, it becomes clear that IGF1R is present in breast cancer cell lines far more frequently than is IGF1 or IGF2. (Table 1). When IGF1 is detected in breast cancer tumor cells by RT-PCR, it is detected through a nested PCR reaction only. IGF2 detection is easily accomplished in an un-nested RT-PCR reaction in MCF7R cells. The IGF1R is easily amplified from MCF7 cells MCF10A cells and MCF10AT cells. It is likely that IGFs affect tumor cell proliferation either via an endocrine effect (growth factor secreted from another organ) or a paracrine effect (growth factor secreted from a neighboring cell type) as opposed to autocrine stimulation of the tumor cells given the infrequent occurrence that significant amounts of IGFs are actually made by tumor cells. IGF2 and IGFR have been successfully amplified from all tumor tissues studied. For IGF2 and IGFR there appear to be no gross differences in the presence of the growth factor and receptor when animals were exposed to estrogen, rhGH or a combination of the two.

Table 1: Results of RT-PCR Amplification for IGF1, IGF2, and IGF1R in Various Breast Cancer and Breast Disease Cell Lines

Cell Line	IGF1	IGF2	IGF1R
MCF7S	absent	absent	present
MCF7R	Present (nested rxn only)	present	present
MDA-MB-231	absent	absent	present
MCF10A	absent	absent	present
MCF10AT	absent	absent	present

Ribonuclease Protection Assays: Quantitation of IGFR and IGF2 from northern analyses were unsuccessful presumably due to the relatively low production of these proteins by the studied tumor cells. Therefore to assess the effect of exogenously administered rhGH or IGF1 on xenograft function at the receptor level, we developed a ribonuclease protection assay to assess and quantitate the IGFR level in the MCF7R xenografts harvested from animals treated with rhGH, IGF1, 17- β estradiol plus IGF1, 17- β estradiol plus rhGH or 17- β estradiol alone. For animals treated with rhGH by

either bolus or continuous infusion, RPA assays for IGFR and IGF2 failed to reveal any upregulation of IGFR or IGF2 under any of the experimental situations in comparison to untreated control animals (Figures XI and XII).

Figure X1: The effect of rhGH on IGF2 expression in MCF7R xenografts grown in NOD *scid/scid* mice, *scid/scid* lit/lit mice, and TghGH *scid/scid* mice

Lanes 1-4: NOD *scid/scid* mice, Lanes 5-8: *scid/scid* lit/lit mice, Lanes 9-10: TghGH *scid/scid* mice. GH=rhGH. +E= 17- β estradiol

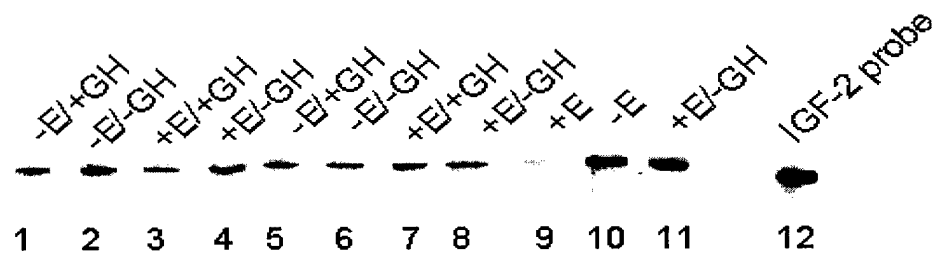
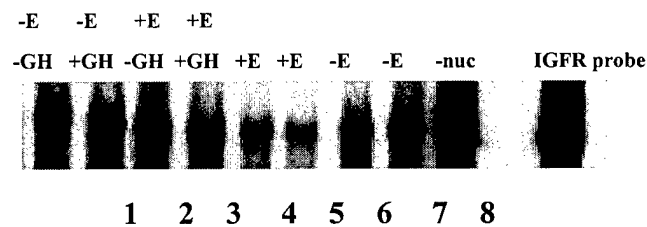


Figure XII: The effect of rhGH administration on IGFR expression in MCF7R xenografts grown in NOD *scid/scid* mice and TghGH *scid/scid* mice

Lanes 1-4: NOD *scid/scid* mice, Lanes 4-8: TghGH *scid/scid* mice



The results of the ribonuclease protection assay for the MCF7R xenografts harvested from experimental animals treated with IGF1 is displayed in Figure XIII. Xenografts harvested from experimental animals treated with any combination of IGF1 and or 17- β estradiol had similar up-regulation of the IGFR in comparison to xenografts treated from untreated control animals. These results clearly demonstrate that IGF1 and 17- β estradiol have an effect on the IGFR but together are not capable of receptor stimulation in an additive fashion. The enhanced xenograft growth documented in animals treated with both IGF1 and 17- β estradiol can not be uniquely attributed to increased signaling through the IGF1R. Other receptors in addition to the ER and IGF1R are therefore likely to be implicated in IGF1 signaling and *in vivo* cell proliferation.

Figure XIII: Quantitation of IGFR by Ribonuclease Protection Assay in MCF7R Xenografts harvested from *Scid/Scid Lit/Lit* (A) and *Scid/Scid Lit +/-* (B) Animals Treated with rhIGF1, and/or 17- β estradiol

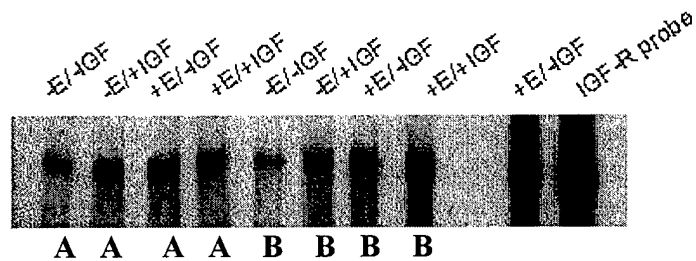


Figure XIV displays the effect of IGF1 treatment on IGF2 expression as quantitated by an RPA for IGF2. In the MCF7R xenografts harvested from animals treated with rhIGF1, we have also evaluated the expression of IGF2 by RPA. When IGF2 is quantitated by RPA, there is little difference in expression of IGF2 under any of the experimental conditions in comparison to xenografts harvested from untreated control animals. IGF2 production by tumor and/or stromal cells in this model system appears to be constitutively activated and not influenced by the presence of high hIGF1 levels or 17- β estradiol supplementation.

Figure XIV: Quantitation of IGF2 by Ribonuclease Protection Assay in MCF7R Xenografts harvested from *Scid/Scid Lit/Lit* (A) and *Scid/Scid Lit +/-* (B) Animals Treated with rhIGF1 (I), and/or 17- β estradiol (E)

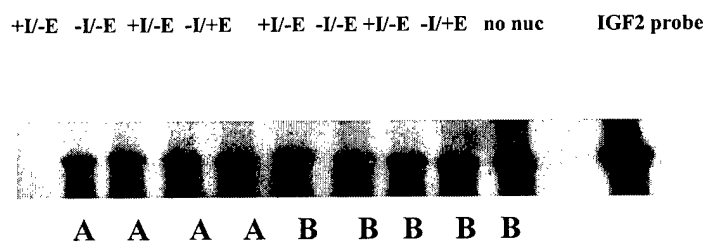
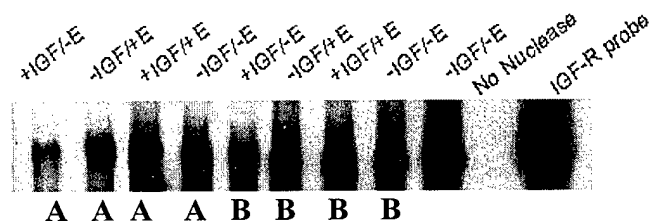


Figure XV displays the effect of rhIGF1 on IGF1R expression in the ER negative xenografts derived from MDA-MB-231 cells. Exogenous IGF1 administration has little if any effect on IGF1R expression in this ER negative cell line. Likewise, 17- β estradiol administration fails to result in any augmentation in IGFR expression. This was a predictable result given that growth factor supplementatiuon in any combination failed to result in enhancement of growth *in vivo* (Figure IX).

Figure XV: Quantitation of IGFR by Ribonuclease Protection Assay in MDA-MB-231 Xenografts harvested from *Scid/Scid Lit/Lit* (A) and *Scid/Scid Lit +/-* (B) Animals Treated with rhIGF1, and/or 17- β estradiol



Specific Aim 4: To grow primary breast cancer explants in the optimized animal model. Unfortunately, when primary xenografts were placed in scid/scid mice and supplemented with continuous infusion of rhIGF1 through Alza miniosmotic pumps, there was no successful engraftment of tissue. This was the case for xenografts placed as “tumor chunks” in the mammary fat pad of the

experimental animals as well as tumors that were placed from single cell suspensions. Clearly, IGF1 supplementation results in robust xenograft maturation for ER positive tumor cells derived from well established cell line tumors. There are still elusive factors not related to IGF biology that are required for primary xenograft take and maturation that were not in the scope of this study.

3.0 CONCLUSIONS

- In the absence of any growth factors, MCF7R cells will form palpable tumors in *scid/scid* mice. The time to engraftment is 6-7 weeks.
- rhGH administered as a bolus or continuous infusion may result in MCF7R tumors that are slightly larger than untreated control animals. This growth enhancement in no way exceeds the effect of 17- β estradiol.
- TghGH *scid/scid* mice bearing MCF7R xenografts have tumors that achieve excessive tumor volume only when under the influence of 17- β estradiol supplementation. MCF7R xenografts grown in TghGH *scid/scid* mice not supplemented with 17- β estradiol have no particular growth advantage in comparison to xenografts grown in NOD *scid/scid* mice. Growth hormone is therefore not a more important growth factor in ER positive tumor engraftment and progression than 17- β estradiol.
- MCF7R xenografts grown in *scid/scid lit/lit* mice under the influence of no growth factors or 17- β estradiol only have reduced tumor sizes at each weekly time point in comparison to *lit+/-* or NOD *scid/scid* mice. Clearly endogenous murine IGF1 is somewhat important in allowing these xenografts to grow into sizable masses.
- MCF7R xenografts grown in *lit/lit scid/scid* mice supplemented with rhIGF1 achieve tumor volumes in excess of those treated with 17- β estradiol alone. Xenografts are first noted at 4 weeks post tumor implantation. These tumors begin to achieve average tumor volumes comparable to xenografts grown in NOD *scid/scid* mice or *lit+/- scid scid* mice. The addition of 17- β estradiol to rhIGF1 supplementation does not appear to augment tumor growth in these animals over and above the effect of IGF1 alone. Addition of rhIGF1 to MCF7R ER positive xenografts grown in *lit/lit* mice is critical to the successful early engraftment and robust growth of tumors in these GH and IGF1 depleted animals.
- IGF1 supplementation and 17- β estradiol supplementation result in the up-regulation of the IGF1R as quantitated by an RPA assay. The two growth factors together do not result in up-regulation of the receptor over and above the effect seen with each growth factor alone.

- RhGH and IGF1 supplementation have little if any effect on IGF2 expression in this experimental work.
- IGF1 does not appear to have a significant effect alone or in combination with 17- β estradiol in MDA-MB-231 ER negative cell growth *in vivo*. Likewise, these growth factors have little effect on IGF1R, or IGF2 expression as measured by RPA.
- Supplementation of primary xenografts with rhIGF1 obtained from patients with breast cancer failed to take in the *scid/scid* mouse model.

4.0 REFERENCES

1. Rygaard, J. and C.O. Povlsen 1969 Heterotransplantation of a human malignant tumour to "nude" mice. *Acta path microbiol scand*, **77**: 758-760.
2. Giovanella, B.C., et al. 1989. Poor prognosis of breast cancer patients whose tumor took in nude mice. Correlation with amplification and overexpression of the Her-1/neu oncogene. *Proc Amer Assn Cancer Res*, **30**: 60.
3. Giovanella, B.C. and J. Foght. 1985. The nude mouse in cancer research. *Adv Cancer Res*, **44**: p.69- 120.
4. Maruo, K., et al..1982. Strain-dependent growth of a human carcinoma in nude mice with different genetic background. *Expl Cell Biol* **50**:115-119.
5. Dagnaes, F. and G. Poulsen 1992. Growth patterns of human breast cancer xenograft T 60 in different strains of nude mice and in scid mice. *Contrib Oncol*, **42**:131-134.
6. Wilhemi, A.E., 1974. Chemistry of growth hormone, in *Endocrinology*, W.H.S. E. Knobil, Editor. Williams & Wilkins Co.: Baltimore. p.59-78.
7. Emerman, J.T., M. Leahy, P.W. Gout, and N. Bruchovsky. 1985. Elevated growth hormone levels in the sera from breast cancer patients. *Horm. Metab. Res.* **17**:421-4.
8. Conte, P.F., G./ Gardin, A. Alama, A. Nicolini, and R. Rosso. 1989. In vivo tumor growth expansion induced by diethylstilbestrol (DES) and recombinant human growth hormone (rhGH) in advanced breast cancer: Experimental data and clinical implication (meeting abstract). In: *Joint NCI-IST Symposium. Third IST International Symposium. Biology and Therapy of Breast Cancer*. September 24-27, 1989, Genoa, Italy, A36.
9. Pollak, M. 1993. Adjuvant Tamoxifen therapy induces major changes in growth hormone and IGF-I physiology. 1993. In: *7th International Conference on the Adjuvant Therapy of Cancer*. March 10-13, Tuscon, Arizona **1993**:36.
10. Gross, G.E., D.H. Boldt, and C.K. Osborne. 1984. Perturbation by insulin of human breast cancer cell cycle kinetics. *Cancer Res.* **44**:3570-5.
11. Owens, P.C., P.G. Gill, N.J. DeYoung et al. 1993. Estrogen and progesterone regulate secretion of insulin-like growth factor binding proteins by human breast cancer cells. *Biochem Biophys Res Commun* **193**(2):467-473.
12. Barni, S., P.Lissoni, F. Brivio, L. Fumagalli, D. Merlini, M. Cataldo, F. Rovelli, and G. Tancini. 1994. Serum levels of insulin-like growth factor I in operable breast

cancer in relation to the main prognostic variables and their perioperative changes in relation to prolactin. *Tumori* **80**:212-5.

13. Berns, E.M., J.G. Klijn, I.L. van Staveren, H. Portengen, and J.A. Foekens. 1992. Sporadic amplification of the insulin-like growth factor I receptor gene in human breast tumors. *Cancer Res.* **52**:1036-9.
14. Papa, V., R. Gliozzo, G.M. Clark, W.L. McGuire, D. Moore, Y. Fujita-Yamaguchi, R. Vigneri, I.D. Goldfine, and V. Pezzino. 1993. Insulin-like growth factor I receptors are overexpressed and predict a low risk in human breast cancer. *Cancer Res.* **53**:3736-40.
15. Lonning, P.E., S.I. Helle, D.C. Johannessen, et al. 1995. Relations between sex hormones, sex binding globulin, insulin-like growth factor-1 and insulin-like growth factor binding protein-1 in postmenopausal breast cancer patients. *Clin Endocrinol* **42**(1):23-30.
16. Pollak, M., T.J. Powles, M. Baum, and N. Sacks. 1993. Tamoxifen used as a chemopreventive agent lowers serum IGF-I levels. In: *Proc. Annu. Meet. Am. Assoc. Cancer Res.* **34**:A1518.
17. Clarke R, Howell A, Anderson E: 1997. Type I insulin-like growth factor receptor gene expression in normal human breast tissue treated with oestrogen and progesterone. *Br J Cancer* **75**:251-257
18. Hankins GR, De Souza AT, Bentley RC et al. 1996. M6P/IGF2R: a candidate breast tumor suppressor gene. *Oncogene* **12**:2003-2009.
19. Oates A, Schumaker L, Jenkins et al. 1998. The mannose 6-phosphate/insulin-like growth factor 2 receptor (M6P/IGF2), a putative breast tumor suppressor gene. *Breast Cancer Res Treat* **47**:269-281.
20. Dunn S, Ehrlich M, Sharp NJH, Reiss K, Solomon G, Hawkins R, Baserga R, and Barret CJ. 1998. A dominant negative mutant of the insulin-like growth factor-1 receptor inhibits the adhesion, invasion, and metastasis of breast cancer. *Cancer Res.* **58**(14):3353-3361.
21. Resnicoff M, Sell C, Rubini M, Coppola D, Ambrose D, Baserga R, Rubin R. 1994. Rat glioblastoma cells expressing an antisense RNA to the insulin-like growth factor-1 (IGF-1) receptor are nontumorigenic and induce regression of wild-type tumors. *Cancer Res.* **54**(8):2218-22.
22. D'Ambrosio C, Ferber A, Resnicoff M, Baserga R. 1996. A soluble insulin-like growth factor I receptor that induces apoptosis of tumor cells *in vivo* and inhibits tumorigenesis. *Cancer Res.* **56**:2206-2212

23. Beamer, W.G., et al.. 1993. Granulosa cell tumorigenesis in genetically hypogonadal-immunodeficient mice grafted with ovaries from tumor susceptible donors. *Cancer Res.* **53**: p. in press, Aug 15.
24. Donahue, L.R. and W.G. Beamer. 1993. Growth hormone deficiency in 'little' mice results in aberrant body composition, reduced insulin-like growth factor-I (IGF-I) and insulin like growth factor binding protein-3(IGFBP-3), but does not affect IGFBP-2, -1, or -4. *J. Endocrinol.*, **136**:91-104.
25. Godfrey, P., et al.. 1993. GHRH receptor of little mice contains a missense mutation in the extracellular domain that disrupts receptor function. *Nature Genetics*, **4**:227-232.
26. Yang XF, Beamer W, Huynh HT, and M. Pollak. 1996. Reduced growth of human breast cancer xenografts in hosts homozygous for the "lit" mutation. *Cancer Res* **56**:1509-1511.
27. Lighten, A.D., K. hardy, R. Winston, and G.E. Moore. 1997. Expression of mRNA for the Insulin-like growth factors and their receptors in human preimplantation embryos. *Mol. Repro. Devel.* **4**:134-139.
28. Ambion, Multi-NPA RNA/DNA/Oligo Probe Protection Assay Kit. Catalog 1428.
29. Weisberg T.F. and Carmody. Unpublished data. Dec. 1999.

Bibliography

To date, there have been no publications or presentations on results of this work. I am currently preparing an abstract to be presented at the June *Era of Hope* meeting in Atlanta. Also, a manuscript is in preparation for submission to "***Cancer Research***".

Personnel

Persons receiving pay from this research project are as follows:

Maine Medical Center Research Institute

Bruce Cahill, Laboratory Technician: Sept. '97-June '98

Mary Beth Carmody, Laboratory Technician:

July '98-Sept. '99

The Jackson Laboratory

Aletha Torrey, Research Assistant: Sept. '97-Sept.'99

STATEMENT OF WORK

Technical Objectives (Specific Aims) 1-3

- | | |
|------------------------------|---|
| Task 1: Months 1-4: | Implant MCF-7R tumor cells into experimental animals
Initiate experiments in Aim 1 with rhGH given by bolus or continuous infusion.
Measure serum levels of GH. |
| Task 2: Months 1-4: | Synthesize probes for detection of hGH and IGF-1 for use in northern and western analyses.
Test probes for efficacy on positive and negative control specimens. |
| Task 3: Months 5-8: | Implant MCF-7R tumor cells into experimental animals.
Initiate experiments in Aim 2 with IGF-1
Measure serum levels of IGF-1 |
| Task 4: Months 5-8: | Determine if additional dose levels of rhGH could optimize results. If so, set-up experimental animals to repeat experiments in Aim 1 at higher or lower dose of rhGH. |
| Task 5: Months 5-8: | Perform northern and western analyses on tumors from animals in Specific Aim 1. Probe with GH probe. |
| Task 6: Months 9-12: | Implant MCF-7R tumor cells into experimental animals.
Initiate experiments in Aim 3 with IGF-1/17- β estradiol. |
| Task 7: Months 9-12: | Determine if additional dose levels of IGF-1 could optimize experimental results. If so, set-up experimental animals to repeat experiments in Aim 1 at higher or lower dose of IGF-1. |
| Task 8: Months 9-12: | Perform northern and western analyses on tumors from animals in Specific Aim 2. with IGF-1 probe. |
| Task 9: Months 13-16: | Perform northern and western analyses on tumors from animals in Specific Aim 3. Probe with IGF-1 probe. |

Task 10: Months 13-16:

Repeat any experiments in Aims 1-3 that could help to further optimize the experimental model

Task 11: Months 13-20

Perform northern and western analyses on animals studied in Task 4,7.

Task 12: Months 15-24:

Implement optimized experimental parameters in animal model. Begin implanting primary breast cancers into optimized animal model.